



## A disruption of *ctpA* encoding carboxy-terminal protease attenuates *Burkholderia mallei* and induces partial protection in CD1 mice

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### ABSTRACT

*Burkholderia mallei* is the etiologic agent of glanders in solipeds (horses, mules and donkeys), and incidentally in carnivores and humans. Little is known about the molecular mechanisms of *B. mallei* pathogenesis. The putative carboxy-terminal processing protease (*CtpA*) of *B. mallei* is a member of a novel family of endoproteases involved in the maturation of proteins destined for the cell envelope. All species and isolates of *Burkholderia* carry a highly conserved copy of *ctpA*. We studied the involvement of *CtpA* on growth, cell morphology, persistence, and pathogenicity of *B. mallei*. A sucrose-resistant strain of *B. mallei* was constructed by deleting a major portion of the *sacB* gene of the wild type strain ATCC 23344 by gene replacement, and designated as strain 23344Δ*sacB*. A portion of the *ctpA* gene (encoding *CtpA*) of strain 23344Δ*sacB* was deleted by gene replacement to generate strain 23344Δ*sacB*Δ*ctpA*. In contrast to the wild type ATCC 23344 or the *sacB* mutant 23344Δ*sacB*, the *ctpA* mutant 23344Δ*sacB*Δ*ctpA* displayed altered cell morphologies with partially or fully disintegrated cell envelopes. Furthermore, relative to the wild type, the *ctpA* mutant displayed slower growth *in vitro* and less ability to survive in J774.2 murine macrophages. The expression of mRNA of *adtA*, the gene downstream of *ctpA* was similar among the three strains suggesting that disruption of *ctpA* did not induce any polar effects. As with the wild type or the *sacB* mutant, the *ctpA* mutant exhibited a dose-dependent lethality when inoculated intraperitoneally into CD1 mice. The CD1 mice inoculated with a non-lethal dose of the *ctpA* mutant produced specific serum immunoglobulins IgG1 and IgG2a and were partially protected against challenge with wild type *B. mallei* ATCC 23344. These findings suggest that *CtpA* regulates *in vitro* growth, cell morphology and intracellular survival of *B. mallei*, and a *ctpA* mutant protects CD1 mice against glanders.

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### 1. Introduction

*Burkholderia mallei*, the causative agent of glanders, is a Gram-negative, aerobic bacillus. Primarily a disease of solipeds, glanders is generally confined to equines (horses, mules and donkeys) [1,2]. Glanders in solipeds presents as a chronic or as an acute disease. The chronic form occurs either as a pulmonary disease, an upper respiratory disease, or a cutaneous disease. The symptoms of the acute form of the disease include high temperature, depression, shortness of breath, diarrhea, and rapid weight loss with mortality [1,2]. Death may occur after a few weeks from an acute infection, whereas the chronic form of glanders may persist for years and may

end in death. Nearly 90% *B. mallei* infections in horses are latent [37], and chronically infected horses serve as reservoirs for transmitting the disease to uninfected animals [3]. *B. mallei* is an obligate animal pathogen that also can infect mice, guinea pigs, rabbits, monkeys, hamsters, and carnivores including lions and dogs that consume contaminated meat [2,4].

In humans, glanders is primarily an occupational disease that affects individuals who have close contact with infected animals [1,2,5]. Infection results primarily from exposure to affected animals via wounds, abrasions or mucous membranes. Glanders in humans is almost always fatal unless treated with antibiotics [33,36,37]. *B. mallei* is a category-B biological agent because it is highly infectious by aerosol [34,35], resulting in a debilitating acute disease in humans [6].

Relatively little is known about the mechanisms of *B. mallei* pathogenesis. Previous reports indicate that a capsular polysaccharide [7], a type III secretion system [8], a quorum sensing

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<b>14. ABSTRACT</b> <p>Burkholderia mallei is the etiologic agent of glanders in solipeds (horses, mules and donkeys), and incidentally in carnivores and humans. Little is known about the molecular mechanisms of B. mallei pathogenesis. The putative carboxy-terminal processing protease (CtpA) of B. mallei is a member of a novel family of endoproteases involved in the maturation of proteins destined for the cell envelope. All species and isolates of Burkholderia carry a highly conserved copy of ctpA. We studied the involvement of CtpA on growth, cell morphology, persistence, and pathogenicity of B. mallei. A sucrose-resistant strain of B. mallei was constructed by deleting a major portion of the sacB gene of the wild type strain ATCC 23344 by gene replacement, and designated as strain 23344DeltasacB. A portion of the ctpA gene (encoding CtpA) of strain 23344DeltasacB was deleted by gene replacement to generate strain 23344DeltasacBDeltactpA. In contrast to the wild type ATCC 23344 or the sacB mutant 23344DeltasacB, the ctpA mutant 23344DeltasacBDeltactpA displayed altered cell morphologies with partially or fully disintegrated cell envelopes. Furthermore, relative to the wild type, the ctpA mutant displayed slower growth in vitro and less ability to survive in J774.2 murine macrophages. The expression of mRNA of adtA, the gene downstream of ctpA was similar among the three strains suggesting that disruption of ctpA did not induce any polar effects. As with the wild type or the sacB mutant, the ctpA mutant exhibited a dose-dependent lethality when inoculated intraperitoneally into CD1 mice. The CD1 mice inoculated with a non-lethal dose of the ctpA mutant produced specific serum immunoglobulins IgG1 and IgG2a and were partially protected against challenge with wild type B. mallei ATCC 23344. These findings suggest that CtpA regulates in vitro growth, cell morphology and intracellular survival of B. mallei, and a ctpA mutant protects CD1 mice against glanders.</p>				
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network [9], and a type VI secretion system [10] are important for the virulence of this bacterium. Molecular characterization of the *in vivo* survival process (or pathogenicity) of *B. mallei* is important because it would provide guidance for the development of measures for the prevention and control of glanders. Information on the effective treatment of infections due to this organism with antibiotic therapy is sparse. Currently, there is no effective animal or human vaccine that provides protection against infection with *B. mallei*.

Inactivation of individual genes by allelic exchange or transposon mutagenesis has been the most precise way of elucidating the specific roles of genes in the pathogenicity of a bacterium. *B. mallei* strain SR1 was previously used as the platform in making genetically-defined deletion mutants of this bacterium [10,11]. Strain SR1 was resistant to sucrose, a feature that was used as a non-antibiotic selection marker in these genetic manipulations. Strain SR1 harbors an IS407A-mediated deletion of a 78-kb region of chromosome II encompassing the *sacB* gene [12]. In this communication, we report the successful construction of an isogenic  $\Delta$ *sacB* strain of *B. mallei* that can also be used as a platform to delete targeted genes. This new strain is sucrose-resistant due to a deletion that specifically removes only the *sacB* gene, leaving the adjacent genes intact.

The proteins destined for extracytoplasmic locations are initially synthesized as precursor forms and processed into mature forms by proteolytic cleavage to remove short peptide sequences, near either the amino terminus or the carboxyl terminus. The endoproteases responsible for cleaving of amino-terminal peptides are called signal peptidases or amino-terminal processing proteases and have been well characterized [13]. A relatively new class of endoproteases with carboxyl-terminal processing activities has been identified from higher plants, algae, and bacteria [14–18]. These carboxyl-terminal proteases (Ctp) are serine proteases that utilize a Ser/Lys catalytic dyad instead of the well-known Ser/His/Asp catalytic triad [19].

The Ctp of *Escherichia coli* is responsible for cleavage of C-terminal 11 amino acid residues of the precursor penicillin-binding protein-3 (PBP-3) [20,21]. A Ctp mutant *E. coli* strain that was defective in PBP-3 processing exhibited filamentous cell morphology, in contrast to the cocco-bacillus morphology of the wild type *E. coli*. These observations confirmed the role of Ctp on PBP-3 processing and thereby on cell morphology of *E. coli* [20,21]. The Ctp protein is also believed to be involved in protection of the bacterium from thermal and osmotic stresses [20,21] and degradation of certain aberrant cellular proteins [22]. In *Borrelia burgdorferi*, Ctp is involved in up- or down-regulation of protein expression [14,16,21]. The putative Ctp protein of *Brucella suis* influences cell morphology, salt-sensitive growth, and *in vitro* and *in vivo* persistence [23]. The genome of *B. mallei* strain ATCC 23344 carries a gene, BMA3209, which shares up to 66% identity at the amino acid level with the Ctps of other bacteria. This putative protein is designated as CtpA. Using isogenic mutant strains, we report the influence of CtpA on growth, cell morphology, *in vitro* persistence, and *in vivo* pathogenicity of *B. mallei*. In addition, we report the successful use of a CD1 mouse model to study the pathogenicity of *B. mallei*.

## 2. Results

### 2.1. Nucleotide and protein sequence of *ctpA*

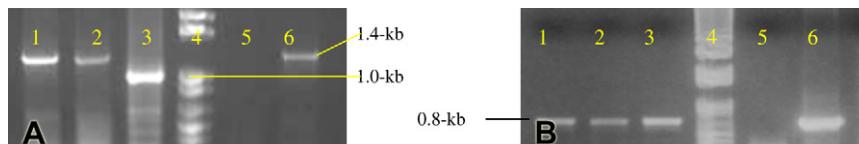
The *ctpA* gene (BMA3209) is 1574-bp long and located on chromosome I of *B. mallei* strain ATCC 23344 (GenBank accession NC\_006348). Just upstream of *ctpA* is *gpmA* (BMA3208) encoding an enzyme catalyzing the interconversion of 2-phosphoglycerate to 3-phosphoglycerate. Downstream of *ctpA* is a gene (BMA3210) encoding an ATP-dependent adenylate transferase (*adtA*), involved in transfer of adenyl moiety to the MoeD subunit of molybdopterin synthase. The DNA sequence analyses predicted that CtpA has a 29 amino acid long N-terminal signal sequence (signal peptide probability: 1.00; maximum cleavage site probability: 0.424 between amino acid positions 29 and 30). The predicted subcellular localization of the putative CtpA protein is the periplasmic space (Reliability Index: RI = 4; Expected Accuracy = 92%). At the amino acid level, *B. mallei* CtpA shared 100% identity with *B. pseudomallei* putative CtpA, 98% identity with *B. thailandensis* CtpA, and up to 92% identity with the putative CtpA of other *Burkholderia* species, including *B. vietnamiensis*, *B. cepacia*, *B. cenocepacia*, and *B. dolosa*. Furthermore, *B. mallei* CtpA also shared considerable identity with the putative CtpAs of other bacterial species including *Brucella*, *Ralstonia*, *Polaromonas*, *Methylibium*, *Bordetella*, *Neisseria*, *Pseudomonas*, and *Legionella*.

### 2.2. Genomic characterization of *sacB* and *ctpA* mutants of *B. mallei*

A  $\Delta$ *sacB* mutant strain of *B. mallei* was constructed by disrupting the *sacB* gene of wild type strain ATCC 23344, and designated as 23344 $\Delta$ *sacB*. A PCR assay with the primer pair Sac5/Sac8 (see Section 5) produced a predicted 3.3-kb amplicon from the wild type *B. mallei* strain ATCC 23344, and an approximately 2.0-kb product from the *sacB* mutant strain 23344 $\Delta$ *sacB* (data not shown), indicating that due to a homologous recombination event, a 1243-bp region was deleted from the *sacB* gene.

A  $\Delta$ *ctpA* mutant strain of *B. mallei* was constructed by disrupting the *ctpA* gene of the mutant 23344 $\Delta$ *sacB*, and designated as 23344 $\Delta$ *sacB* $\Delta$ *ctpA*. A PCR assay with the primer pair CtpA-1/CtpA-4 (see Section 5) produced a predicted 1.3-kb amplicon from wild type *B. mallei*, and an approximately 1.0-kb amplicon from the *ctpA* mutant strain 23344 $\Delta$ *sacB* $\Delta$ *ctpA* (data not shown), indicating that due to homologous recombination event, a 299-bp region was deleted from the *ctpA* gene. Sequencing data suggest that due to the recombination event, the *ctpA* mutant lost 299-bp region in the middle of the *ctpA* gene. In addition, this recombination event produced a frame-shift at the 3' end of the deletion site, but no stop codons were introduced as a result of this event. While the wild type, the *sacB* mutant, and the *ctpA* mutant were resistant to polymyxin and sensitive to gentamicin, both mutants were resistant to sucrose.

Reverse-transcription (RT)-PCR results suggest that both the wild type and the *sacB* mutant expressed a full-length *ctpA* mRNA (lanes 1 and 2 of Fig. 1A). As a result of the homologous recombination event in the *ctpA* gene, the *ctpA* mutant expressed a *ctpA* mRNA that was nearly 0.3-kb shorter than the full-length mRNA (lane 3 of Fig. 1A). In order to characterize any polar effect



**Fig. 1.** Expression of *ctpA* (A) and *adtA* (B) mRNA by *B. mallei* strains as determined by reverse-transcription PCR. For PCR assays, cDNA from the wild type (lane-1), *sacB* mutant (lane-2), or *ctpA* mutant (lane-3) was used. Genomic DNA from the wild type (lane-6), and DNase-digested genomic DNA from the wild type (lane-5) were used as controls in PCR. Primers AdtA-Forward and AdtA-reverse were used in PCR with *adtA*, whereas, CtpA-1 and CtpA-4 were used with *ctpA*. Lane-M represents 1-kb molecular size marker.

induced by this recombination event, the expression of mRNA of *adtA* was analyzed. The gene *adtA* was chosen for this assay since it is located immediately downstream of *ctpA* in *B. mallei* genome. Just like the wild type and the *sacB* mutant strains, the *ctpA* mutant produced an approximately 0.7-kb amplicon in RT-PCR (lanes 1–3 of Fig. 1B), suggesting that expression of *adtA* mRNA was not affected due to the homologous recombination event in this mutant.

The total RNA used for RT-PCR assays were pre-treated with RNase-free DNase to get rid of any residual DNA. To assess the efficiency of the RNase-free DNase treatment, the genomic DNA from the wild type strain was treated with this enzyme and used in PCR under the same experimental conditions. The wild type genomic DNA that was not pre-treated with RNase-free DNase produced expected size amplicons (lane 6, Fig. 1A,B), whereas, the genomic DNA pre-treated with RNase-free DNase failed to produce any amplicon (lane 5, Fig. 1A,B). These observations suggest that DNase pre-treatment totally and efficiently removed any of the contaminating DNA in the extracted RNA.

### 2.3. Growth rates of recombinant *B. mallei* strains

The growth of *B. mallei* strains in trypticase soy broth supplemented with 4% glycerol (TSB-G) or salt-free Luria–Bertani broth supplemented with 4% glycerol (LB-G) was measured. In TSB-G, during log phase, the growth of the *ctpA* mutant was slower (approximately 3.5 h doubling time) than that of the wild type or the *sacB* mutant (approximately 1.75 h doubling time) (data not shown). In salt-free LB-G that has been shown to stress other *ctpA* mutants, the growth rate of the *B. mallei* *ctpA* mutant was approximately 2-fold slower than the *B. mallei* wild type or *sacB* mutant strains (data not shown).

### 2.4. Cell morphology

When examined by electron microscopy, the wild type strain (Fig. 2A), and the *sacB* mutant (Fig. 2B) grown in TSB-G displayed the native cocco–bacillus shape of *Burkholderia*. In contrast, the *ctpA* mutant strain grown in the same media acquired various cell shapes, and the cell envelopes appeared more unorganized (Fig. 2C).

### 2.5. Persistence of *B. mallei* strains in J774.2 macrophages

The survival of *B. mallei* strains in J774.2 murine macrophage cell line was measured. The recovery of strains from murine macrophages (J774.2) 4 h post-inoculation was estimated in terms of colony forming units (cfus):  $3.85 \pm 0.15$  for the wild type,  $3.91 \pm 0.27$  for the *sacB* mutant and  $2.81 \pm 0.36$  for the *ctpA* mutant. The results show a  $1.04 \log_{10}$  decline of the *ctpA* mutant strain compared to the wild type or the *sacB* mutant strains by 4 h post-inoculation in murine macrophages.

### 2.6. Pathogenicity in mice of the *B. mallei* strains

The pathogenicity of the *B. mallei* strains in CD1 mice was evaluated in two separate trials. The results of a representative trial are presented here. When approximately  $4.4 \times 10^5$  cfu of wild type *B. mallei* were used for intraperitoneal (i.p) inoculations, 20% of CD1 mice died, but no mice inoculated with the *sacB* or *ctpA* mutants died at this dose (Fig. 3A). When a larger dose of inoculum ( $6.6 \times 10^5$  cfu) was used, 60% of animals inoculated with the wild type or *ctpA* mutant died within 4 days post-inoculation, and 40% animals inoculated with the *sacB* mutant died within 6 days post-inoculation (Fig. 3B). In contrast, only 20% of animals inoculated with the same dose ( $6.6 \times 10^5$  cfu) of an attenuated *B. mallei* strain (wzt mutant – control strain) died (Bandara et al., 2008,

unpublished data). When the mice were inoculated with  $8.8 \times 10^5$  cfu, all mice injected with the wild type, *sacB* mutant, or *ctpA* mutant died within 4 days post-inoculation (Fig. 3C). In contrast, only 40% of animals inoculated with the same dose ( $8.8 \times 10^5$  cfu) of the attenuated wzt mutant *B. mallei* strain died (Bandara et al., 2008, unpublished data). The LD<sub>50</sub> dose of the wild type ATCC 23344, the *sacB* mutant, and the *ctpA* mutant strains were  $5.9 \times 10^5$ ,  $6.6 \times 10^5$ , and  $6.2 \times 10^5$  cfu, respectively. In contrast, the LD<sub>50</sub> of the attenuated wzt mutant strain (control) was  $9.1 \times 10^5$  cfu (Bandara et al., 2008, unpublished data). None of the inoculated mice died after 6 days post-inoculation. Those mice that survived the *B. mallei* inoculations exhibited clinical manifestations including huddling during the first 4 days following inoculations, but remained clinically normal throughout the rest of the 36-day study.

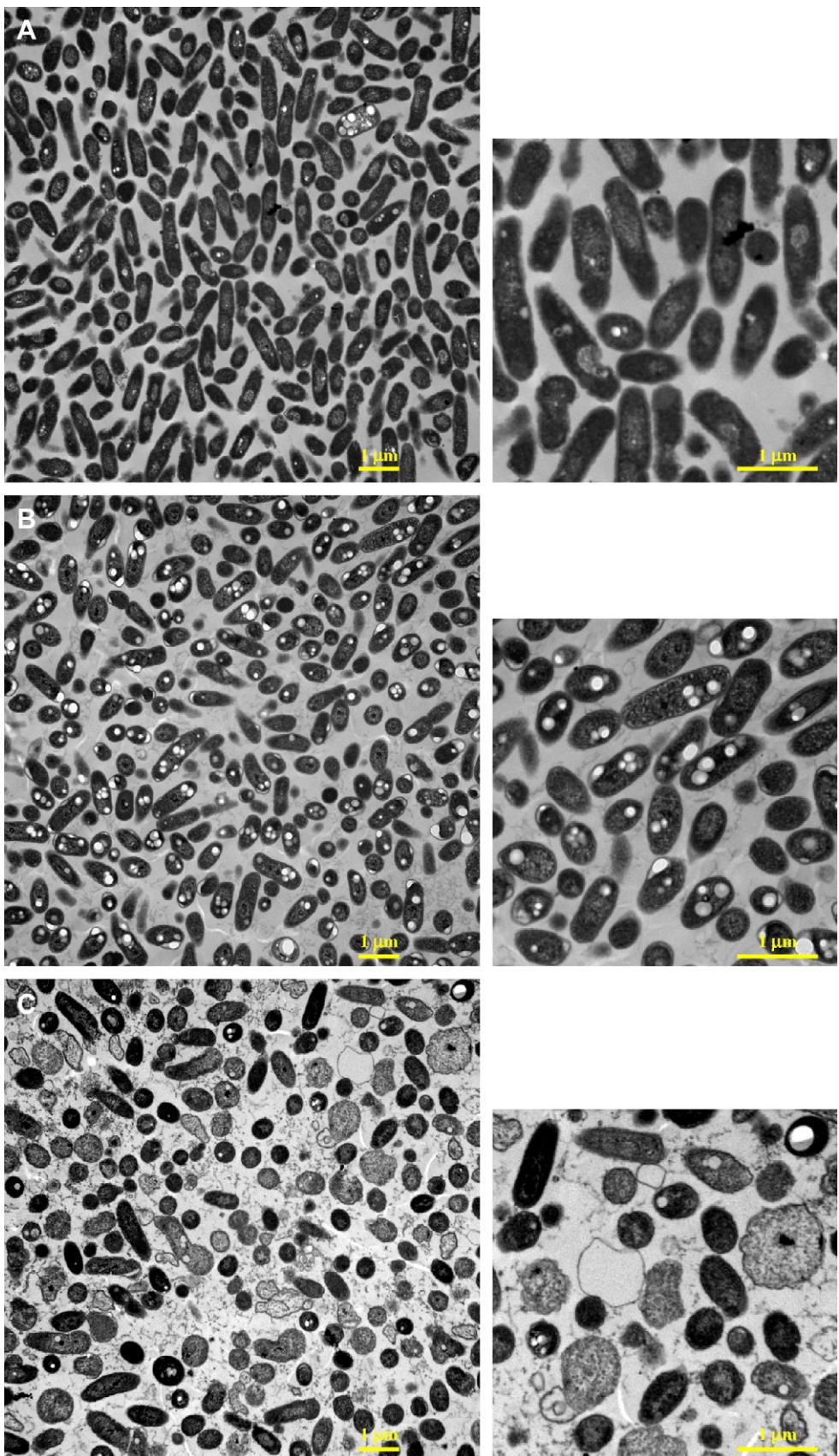
### 2.7. Immune and protective responses of mice inoculated *ctpA* mutant *B. mallei*

Specific serum immunoglobulins IgG1 and IgG2a of CD1 mice were measured by enzyme-linked immunosorbent assay. Serum IgG titers of mice inoculated with the *ctpA* mutant were 31–42-fold higher than naïve controls at 30 days post-infection. The two IgG isotypes were present in sera in almost equal amounts. The protective efficacy of the *ctpA* mutant in CD1 mice against challenge with the *B. mallei* wild type ATCC 23344 was determined. At day 36 post-inoculation, those mice that were injected with saline or the *ctpA* mutant and survived were challenged intraperitoneally with  $6.6 \times 10^5$  cfu of strain ATCC 23344, and behaviors and survival of mice were monitored. Seventy five percent of mice injected with saline and subsequently challenged with strain ATCC 23344 died within 3 days post-challenge (Fig. 4). Gross pathological analysis of spleen extracts from surviving animals (sacrificed on day 15 post-challenge) revealed severe splenomegaly with severe splenic abscesses (Fig. 5D). When the homogenized tissue samples from livers and spleens of the sacrificed animals were plated on trypticase soy agar supplemented with 4% glycerol (TSA-G), viable *B. mallei* were recovered from spleens, but not from livers (data not shown).

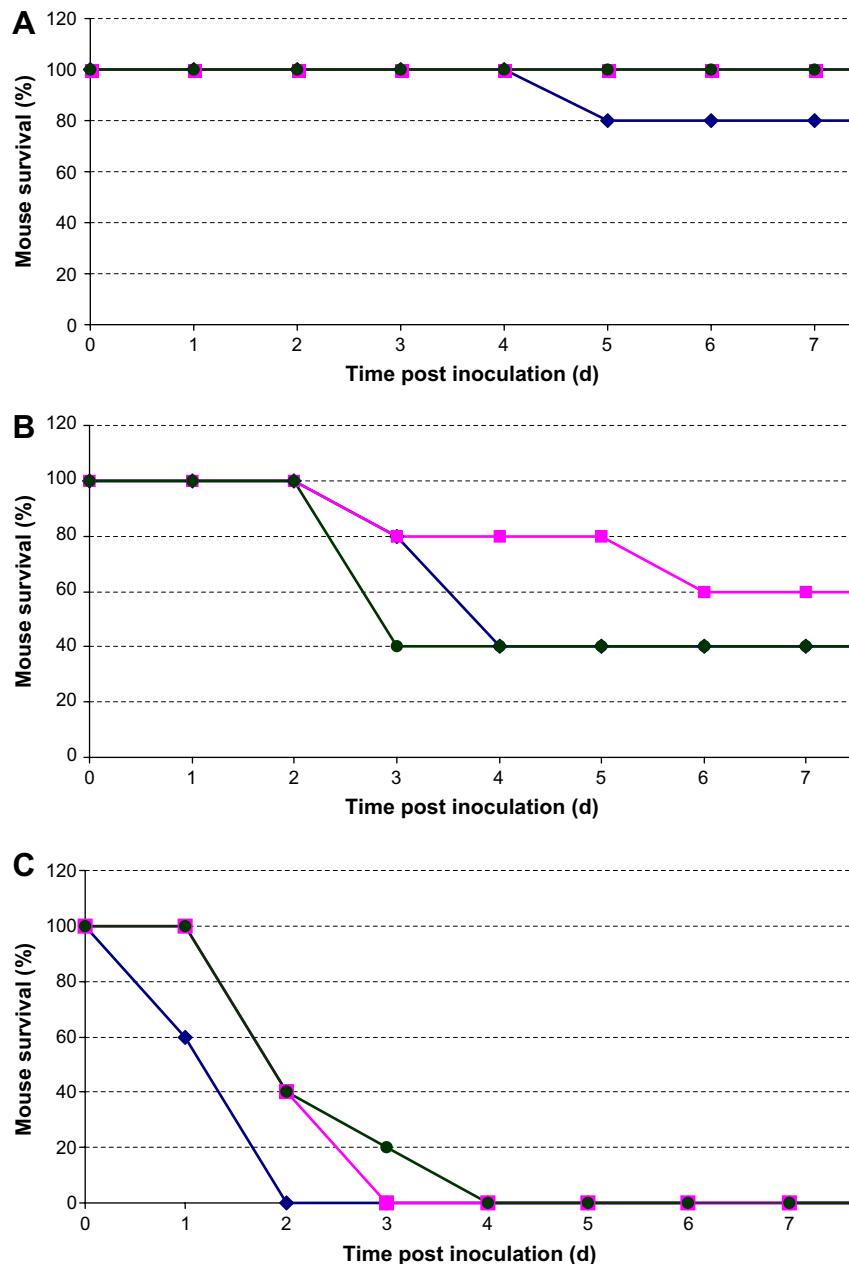
Seventy five percent of the mice inoculated intraperitoneally with  $4.4 \times 10^5$  cfu of the *ctpA* mutant and subsequently challenged intraperitoneally with  $6.6 \times 10^5$  cfu of strain ATCC 23344 survived (Fig. 4). The surviving mice did not exhibit any clinical symptoms during the 15-day post-challenge period. However, gross pathological analyses of spleen extracts from surviving animals (15 days post-challenge) revealed moderate (Fig. 5B) or severe (Fig. 5C,D) splenomegaly, with moderate (Fig. 5C) to severe (Fig. 5D) splenic abscesses. The spleens with severe splenomegaly carried a greater number of viable *Burkholderia*, whereas, those with moderate splenomegaly produced fewer numbers of *Burkholderia* on TSA-G plates (data not shown). The livers did not carry any viable *B. mallei*. Control mice that were not inoculated with *B. mallei* did not display any splenomegaly or abscesses (Fig. 5A).

### 3. Discussion

This investigation analyzed the role of CtpA of *B. mallei* on growth, cell morphology, intracellular persistence and pathogenicity in mice. A sucrose-resistant strain of *B. mallei*, SR1, which harbors an IS407A-mediated deletion in *sacB* gene has been used in past as the platform in making other mutants [10,11]. We constructed an isogenic *ΔsacB* mutant strain of *B. mallei* that can also be used as a platform to delete targeted genes. The new *sacB* mutant strain described here has an advantage over strain SR1 in that it is sucrose-resistant due to a deletion that specifically removes only the *sacB* gene, leaving the adjacent genes intact. Furthermore, unlike strain SR1, the *sacB* mutant strain was



**Fig. 2.** Cell morphology of *B. mallei* as observed by electron microscopy. The wild type (A) and the *sacB* mutant (B) strains displayed native cocco-bacillus morphology, whereas, the *ctpA* mutant (C) displayed various cell morphologies with disintegrated cell envelopes. Electron micrographs with 10,000 $\times$  magnification and 20,000 $\times$  amplified frames are provided.



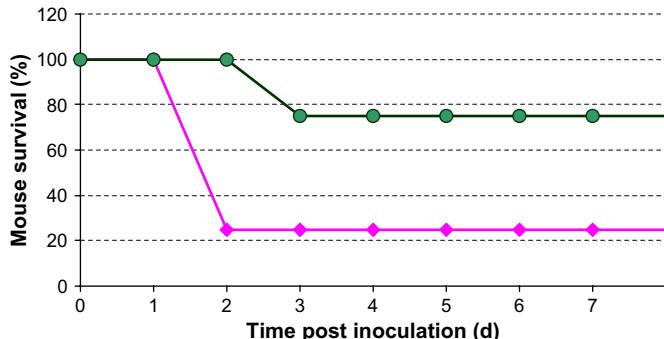
**Fig. 3.** Survival of CD1 mice inoculated *B. mallei* strains wild (◆), *sacB* mutant (■), and *ctpA* mutant (●). Groups of five mice were injected intraperitoneally with  $4.4 \times 10^5$  (A),  $6.6 \times 10^5$  (B), or  $8.8 \times 10^5$  (C) cfu/mouse, and the number of survivors was recorded during a course of 7 days post-inoculation.

constructed by gene replacement and can therefore be considered as a genetically-defined strain. Genetic characterization of a mutant is important in case the mutant is used as the platform for making live vaccine strains. The *sacB* mutant was not different from the wild type strain in terms of *in vitro* growth or persistence in macrophages, or *in vivo* pathogenicity in mice (details to follow), making it an ideal platform strain to generate other isogenic mutants of *B. mallei*. With the construction of the isogenic *ctpA* mutant, we confirmed that the *sacB* mutant can be used as the platform for generating other mutants of *B. mallei*.

The *B. mallei* CtpA shared up to 100% identity with the Ctps of other pathogenic and non-pathogenic *Burkholderia* species. These included *B. pseudomallei* that causes the glanders-like disease melioidosis in humans [24–26], and the non-pathogenic soil saprophyte *B. thailandensis* [27]. Based on this substantial identity among different Ctps, it is possible that this protein provides the same function(s) among of all *Burkholderia* species. The *B. mallei*

CtpA also shared up to 66% identity with Ctp proteins of a vast number of other bacterial species suggesting that this protein is conserved and may be important for the growth and/or persistence of many bacteria.

The *B. mallei* CtpA was predicted to localize in the periplasmic space of the cell similar to that predicted for the CtpA of *Brucella* [23] and *E. coli* [20,21]. The *ctpA* mutant *B. mallei* displayed various cell morphologies including disintegrated cell membranes, suggesting that *ctpA* function is important for the integrity of the cell envelope. A similar phenomenon was seen when *ctpA* was disrupted in *Brucella* [23]. In *E. coli*, Ctp-deficient strains fail to process PBP proteins and thereby display altered cell morphology [20,21]. Further work is needed to find out if CtpA is involved in processing any PBP of *B. mallei*. Just like the *ctpA* mutant *Brucella* [23], the *ctpA* mutant of *B. mallei* displayed slower growth *in vitro*, suggesting that CtpA functions are important for normal growth. However, unlike the *ctpA* mutant *Brucella* [23] or *E. coli* [20,21], the



**Fig. 4.** Protective efficacy of the *ctpA* mutant against challenge with virulent wild type *B. mallei*. Thirty-six days after injection with saline (◆) or inoculation with the *ctpA* mutant (●), the mice that survived were challenged intraperitoneally with  $6.6 \times 10^5$  cfu/mouse of the wild type strain ATCC 23344. The number of survivors was recorded during a course of seven days post-challenge.

*ctpA* mutant *B. mallei* did not exhibit zero-growth in salt-free media, suggesting that CtpA is dispensable for the protection of *B. mallei* from osmotic stresses.

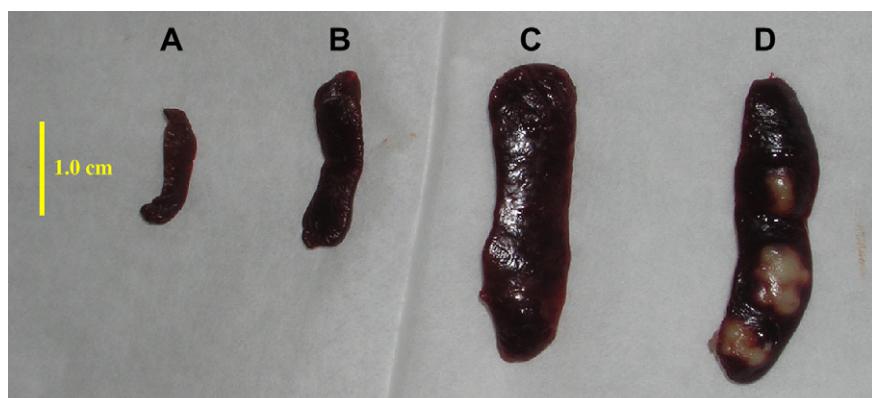
Compared with the wild type strain or the *sacB* mutant, the *ctpA* mutant displayed reduced persistence in J774.2 macrophages at 4 h post-infection. These results suggest that CtpA is important for the intracellular survival of *B. mallei* in J774.2 macrophages. We previously reported that CtpA is important for the intracellular survival of *Brucella* species in J774.2 macrophages [23]. However, when CD1 mice were inoculated intraperitoneally, the *B. mallei* *ctpA* mutant caused murine mortality at a similar rate as that of the wild type. These observations suggest that functions of CtpA may not be critical for the pathogenicity of *B. mallei* *in vivo*. The slower *in vitro* growth was apparently not related to *in vivo* pathogenicity of the *ctpA* mutant. In this aspect, *B. mallei* CtpA seems different from *Brucella* CtpA, where the latter is important for *in vivo* persistence of *Brucella* in BALB/c mice [23]. The disruption of *ctpA* did not affect expression of the genes located downstream. Thus, the differences of the *ctpA* mutant with the wild type or the *sacB* mutant in terms of cell morphology and *in vitro* persistence can be the results of deletion effects, but not the polar effects.

Currently, no vaccines exist for preventing glanders in humans or animals. Since *B. mallei* is a facultative intracellular pathogen, a live attenuated vaccine may be the best strategy to induce protective cell-mediated and antibody-mediated immune responses. Attenuated vaccine strains often establish limited infections that mimic natural infections. The attenuated strain facilitates induction

of systemic and mucosal immune responses and often the production of cytokines that recruit elements of the immune system that ordinarily do not respond to subunit and inactivated vaccines. When the CD1 mice were inoculated with a non-lethal dose of *ctpA* mutant, both IgG1 and IgG2a were induced. These two immunoglobulins were found in almost equal proportions suggesting that both Th1 and Th2 responses were triggered. Ulrich et al. [11] showed that when BALB/c mice were inoculated aerogenically with a non-capsulated mutant of *B. mallei*, serum IgG1 and IgG2a are induced in equal proportions, but when the vaccinated animals were boosted (revaccinated), a greater proportion of serum IgG1 was elicited. In the same study, when BALB/c mice were aerogenically inoculated with a branched-chain amino acid auxotroph, a greater proportion of serum IgG2a was induced. Amemiya et al. [28] reported that parenteral vaccination of BALB/c mice with non-viable *B. mallei* elicited a Th2-like Ig subclass antibody response. These findings suggest that the type of immune response probably varies with the animal model, route of inoculation, and type of inoculum or mutant strain used in vaccination.

When CD1 mice that received a non-lethal dose of the *ctpA* mutant were subsequently challenged with a lethal dose of the wild type, a greater proportion survived relative to uninoculated controls suggesting that the mutant induces protection in mice against glanders. The animals that survived after *ctpA* inoculation and subsequent challenge with strain ATCC 23344 did not exhibit prolonged clinical symptoms like huddling or fur ruffling. Nevertheless, these surviving animals displayed mild to severe splenomegaly with mild to severe abscesses even 15 days after challenge. These observations suggest that although inoculations with non-lethal doses of *ctpA* mutant protect a greater proportion of animals against glanders, this protection is only partial. We were unable to test the protection against an aerogenic challenge, due to non-availability of aerosol infection facilities. During the course of our study we found that intranasal inoculation of mice (by administering drops of bacterial culture into the nose of anesthetized mice) fails to produce consistent results.

The lack of effective and inexpensive animal models and convenient inoculation techniques, have been barriers to study the pathogenicity of certain bacterial species. In the past, Syrian hamsters had been the most effective model to study the pathogenicity of *B. mallei* when infected intraperitoneally [7,8,9,29]. However, purchasing and handling of Syrian hamsters is costlier than mice. The BALB/c mouse model has been effective only when the inoculations were performed by aerosol [7,8,11]. Our results suggest that relatively inexpensive CD1 mice can be used to study *B. mallei* pathogenicity using intraperitoneal inoculation.



**Fig. 5.** Splenomegaly and splenic abscesses of CD1 mice surviving after injection with saline or CtpA mutant, and subsequently challenged with wild type ATCC 23344. (A) A spleen from a mouse with no *B. mallei* injection; (B) a spleen with moderate splenomegaly with no abscesses; (C) a spleen with severe splenomegaly containing moderate abscesses; and (D) a spleen with severe splenomegaly containing severe abscesses.

## 4. Conclusions

The sucrose-resistance of the *sacB* mutant *B. mallei* strain was found to be useful in generating isogenic mutant strains of *B. mallei*. All species and isolates of *Burkholderia* possess an almost identical copy of the *ctpA* gene. *ctpA* seems important for the *in vitro* growth and persistence of *B. mallei*, but this gene was not critical for pathogenicity in CD1 mice. Inoculation with a non-lethal dose of the *ctpA* mutant induced IgG antibodies and partial protection in CD1 mice against challenge with virulent wild type *B. mallei*. Relatively inexpensive CD1 mice can be viewed as a promising animal model for studying the pathogenicity of *B. mallei* using the intraperitoneal route of inoculation.

## 5. Materials and methods

### 5.1. DNA and protein sequence analyses

The nucleotide sequence of the *B. mallei* *ctpA* gene encoding the CtpA protein was analyzed with DNASTAR software (DNASTAR, Inc., Madison, WI). The presence of any signal sequence of the *B. mallei* CtpA protein was predicted by using the SignalP 3.0 server of the Technical University of Denmark (<http://www.cbs.dtu.dk/>) [30]. The destination of the CtpA protein upon translation and processing was predicted using the Subloc v1.0 server of the Institute of Bioinformatics of the Tsinghua University (<http://www.bioinfo.tsinghua.edu.cn/>). The identity of *B. mallei* CtpA to proteins of the EMBL/GenBank/DDBJ databases was analyzed using the BLAST software [31] at the National Center for Biotechnology Information (Bethesda, MD).

### 5.2. Bacterial strains, plasmids, and reagents

*B. mallei* strain ATCC 23344 was obtained from the Centers for Disease Control and Prevention (CDC). *B. mallei* strains were grown in trypticase soy broth (TSB) or trypticase soy agar (TSA) supplemented with 4% glycerol (Difco Laboratories, Sparks, MD) at 37 °C in the presence of 5% CO<sub>2</sub> as previously described [7]. All experiments with live *B. mallei* were performed in a Biosafety Level 3 facility in the Infectious Disease Unit of the Virginia-Maryland Regional College of Veterinary Medicine per CDC-approved standard operating procedures. *E. coli* XL1Blue was used for general cloning, and *E. coli* S17-1 [38] was used as a mobilizing strain for constructing mutants. The suicide vector pGRV2 [11] that carries the counter-selectable marker *sacB* was employed in generating the

mutant *B. mallei* strains. The plasmids used in this study are listed in Table 1. Bacteria containing plasmids were grown in the presence of gentamicin at 5 µg/ml.

Genomic DNA from *B. mallei* strain ATCC 23344 and plasmid DNA from recombinant *E. coli* strains were harvested by use of kits obtained from Qiagen (Qiagen Inc., Valencia, CA). Restriction digests, Klenow reactions, and ligations of DNA were performed using standard procedures [39]. Restriction enzymes, Klenow fragment, and T4 DNA ligase enzyme were purchased from Promega Corporation (Madison, WI).

### 5.3. Construction of a *ΔsacB* mutant strain of *B. mallei*

PCR primers were designed to amplify the 5' and 3' ends of the *sacB* gene (BMAA0466) on chromosome II of strain ATCC 23344. The primer sequences (5'-3') were as follows: Sac5, ATGGTTGTG TCGACGACGATC; Sac6, AGGGCCAAACGTTGTGTCG; Sac7, CGTACT CGCACTACGTGATG; and Sac8, CGCCGTCATCATCCAGTAAC. PCR amplifications were performed in a final reaction volume of 100 µl containing 1X Taq PCR Master Mix (Qiagen, Valencia, CA), 1 µM oligodeoxyribonucleotide primers, and approximately 200 ng of ATCC 23344 genomic DNA. PCR cycling was performed using a PTC-150 MiniCycler with a Hot Bonnet accessory (MJ Research, Inc., Reno, NV) and heated to 97 °C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) and one cycle at 72 °C for 10 min. The PCR amplicons using the Sac5/Sac6 primer pair was 988-bp, and Sac7/Sac8 primer pair was 979-bp (data not shown).

The PCR products were purified using Quantum Prep PCR Spin Columns (BioRad) and cloned into pCR2.1-TOPO (Invitrogen). The Sac7/Sac8 product was liberated with EcoRI and cloned into the corresponding site in pGRV2. Subsequently, the Sac5/Sac6 product was liberated with XbaI + BamHI and cloned into the corresponding sites in the pGRV2 derivative containing the Sac7/Sac8 product. The orientation of the products in pGRV2 was confirmed by DNA sequencing. The resulting plasmid was termed pDD159.

Plasmid pDD159 was introduced into competent *E. coli* S17-1 cells by electroporation, and the recombinant S17-1(pDD159) colonies were picked from TSA plates containing gentamicin 5 µg/ml. The plasmid pDD159 was delivered to *B. mallei* ATCC 23344 via conjugation with S17-1(pDD159) by using a membrane filter mating technique as follows. Strain S17-1(pDD159) was inoculated into 2 ml of TSB and grown at 37 °C for 18 h with shaking at 200 rpm. *B. mallei* ATCC 23344 was also grown under these conditions in TSB supplemented with 4% glycerol (TSB-G). One hundred µl of each

**Table 1**  
Description of the plasmids and bacterial strains used in this study

Plasmid or strain	Description	Source or reference
<i>Plasmids</i>		
pGRV2	Gene replacement vector, 7.0-kb, Gm <sup>r</sup>	[11]
pDD159	pGRV2 with 5' and 3' ends of <i>B. mallei</i> <i>sacB</i> ; Gm <sup>r</sup>	This study
PAB <i>ctpA</i>	pGRV2 with 5' and 3' ends of <i>B. mallei</i> <i>ctpA</i> cloned at Clal + Ncol of pGRV2; Gm <sup>r</sup>	This study
<i>E. coli</i> strains		
XL1-Blue	General cloning <i>supE44</i> <i>hsdR17</i> <i>recA1</i> <i>gyrA96</i> <i>thi</i> <i>relA1</i> <i>lacF</i> [ <i>proAB</i> <sup>+</sup> <i>lacI</i> <sup>q</sup> <i>lacZ</i> [ <i>Delta</i> <i>M15</i> <i>Tn10</i> ( <i>Tet</i> <sup>r</sup> )]	A.B. Bandara
XL1-Blue(pDD159)	XL1-Blue carrying pDD159; <i>Tet</i> <sup>r</sup> ; Gm <sup>r</sup>	This study
XL1-Blue(pAB <i>ctpA</i> )	XL1-Blue carrying pAB <i>ctpA</i> ; <i>Tet</i> <sup>r</sup> ; Gm <sup>r</sup>	This study
S17-1	Mobilizing strain; transfer genes of RP4 integrated in chromosome; Sm <sup>r</sup> ; Pm <sup>s</sup>	[38]
S17-1(pDD159)	S17-1 carrying pDD159; Sm <sup>r</sup> ; Pm <sup>s</sup> ; Gm <sup>r</sup>	This study
S17-1(pAB <i>ctpA</i> )	S17-1 carrying pAB <i>ctpA</i> ; Sm <sup>r</sup> ; Pm <sup>s</sup> ; Gm <sup>r</sup>	This study
<i>B. mallei</i> strains		
ATCC 23344	Type strain; isolated in 1944 from a human case of glanders; Pm <sup>r</sup> ; Gm <sup>s</sup>	[26]
23344Δ <i>sacB</i>	Strain ATCC 23344 with deletion in <i>sacB</i> gene; Pm <sup>r</sup> ; Gm <sup>s</sup> ; Sucrose <sup>r</sup>	This study
23344Δ <i>sacB</i> Δ <i>ctpA</i>	Strain 23344Δ <i>sacB</i> with deletion in <i>ctpA</i> gene; Pm <sup>r</sup> ; Gm <sup>s</sup> ; Sucrose <sup>r</sup>	This study

culture was added to 3 ml of sterile 10 mM MgSO<sub>4</sub>, mixed, and filtered through a 0.45-μm-pore-size nitrocellulose filter. The filters were placed on TSA plates supplemented with 10 mM MgSO<sub>4</sub> and 4% glycerol, and incubated for 12 h at 37 °C in the presence of 5% CO<sub>2</sub>. The filters were washed with 4 ml of sterile 1× phosphate buffered saline (PBS), and 100 μl aliquots were spread onto TSA plates containing 4% glycerol (TSA-G), 5 μg/ml gentamicin, and 15 μg/ml polymyxin. Gentamicin-resistant (Gm<sup>r</sup>) and polymyxin resistant (Pm<sup>r</sup>) transconjugants were identified after 48 h incubation at 37 °C in the presence of 5% CO<sub>2</sub>. Polymyxin was used to counterselect *E. coli* and gentamicin was used to select for transconjugants containing pDD159 integrated into the chromosome of strain ATCC 23344 via homologous recombination. One of the ATCC 23344::pDD159 colonies was used to inoculate TSB-G. Ten-fold dilutions of the overnight culture were spread onto two types of media: TSA-G or TSA-G + 5% sucrose. Sucrose-resistant colonies were nearly 10<sup>3</sup>-fold less abundant than sucrose-sensitive colonies. Six sucrose-resistant colonies were screened by PCR for the deletion in the *sacB* gene (data not shown). One of the colonies carrying the deletion was chosen for further work and designated 23344Δ*sacB*.

#### 5.4. Construction of a Δ*ctpA* mutant strain of *B. mallei*

PCR primers were designed to amplify the 5' and 3' ends of the *ctpA* gene (BMA3209) of strain ATCC 23344. The primer sequences (5'-3') were as follows: CtpA-1, CGGCCATGGATGCGTATGAAATTG AAG; CtpA-2, CGGATCGATACCTTCGTGCCGGGCTCG; CtpA-3, CGGAT CGATCGACGAACGCCAGATCC; and CtpA-4, CGGCCATGGGAGGATG CGCAACTGCTC. The 5' end of CtpA-1 and CtpA-4 primers carried NcoI sites (in bold case), whereas the 5' end of CtpA-2 and CtpA-3 primers carried Clal sites. The PCR amplifications were performed as described above. The PCR amplicons resulting from the CtpA-1/CtpA-2 primer as well as CtpA-3/CtpA-4 primer pair were 500-bp each (data not shown).

The PCR products were purified, and digested with restriction enzymes Clal and NcoI, and cloned into the Clal-NcoI digested plasmid pGRV2 [11]. This facilitated the directional cloning of PCR products representing the 5' and 3' ends of the *ctpA* gene into pGRV2. The ligation product was transformed into competent *E. coli* XL1Blue cells by electroporation and then screened using standard methods [39]. The XL1Blue colonies carrying recombinant pGRV2 were picked from TSA plates containing 5 μg/ml of gentamicin. The recombinant pGRV2 plasmid constructs from the XL1Blue colonies were harvested, and the presence of correct clones was verified by digesting plasmid DNA with NcoI and/or Clal (data not shown). A recombinant pGRV2 plasmid with directional cloning of *ctpA* products was chosen for further work and designated pAB*ctpA*. The plasmid pAB*ctpA* was introduced into competent *E. coli* S17-1 cells by electroporation, and the recombinant S17-1(pAB*ctpA*) colonies were picked from TSA plates containing 5 μg/ml of gentamicin. The plasmid pAB*ctpA* was delivered to sucrose-resistant *B. mallei* strain 23344Δ*sacB*, by using a membrane filter mating technique, as described above. Six sucrose-resistant colonies were screened by PCR for the deletion in the *ctpA* gene (data not shown). One of the colonies carrying the deletion was chosen for further work and designated 23344Δ*sacB*Δ*ctpA*.

#### 5.5. RNA isolation and reverse transcription-PCR (RT-PCR)

Total RNA was extracted using an RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. RNA was resuspended in RNase-free DNase-free water (Qiagen). In order to get rid of any residual DNA, aliquots of eluted RNA were digested with RNase-free DNase (Qiagen): 5 μg of eluted RNA with 2 units of DNase in 1× DNase buffer for 30 min at RT. The RNA quantity was determined

using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) at 260 nm. cDNA was prepared from 3 μg total RNA in 10 μl final volume using the oligo(dT)<sub>20</sub> primer and other standard components included with the SuperScript™ III First-Strand synthesis system (Invitrogen). The resulting cDNA served as a template for PCR amplification of either 0.8-kb of *adtA* or 1.4-kb of *ctpA* as a control. The final PCR mixture contained 3 μl of cDNA, 1× PCR buffer (New England Biolabs), 0.2 mM deoxynucleoside triphosphates, 1 units of Taq DNA polymerase (New England Biolabs), and 0.8 μM sense and antisense primers. The primers AdtA-Forward (5' ATGAACGACCAACAATCCT 3') and AdtA-Reverse (5' TGTTCCATTCCATCCGCAGC 3') were used for PCR amplification of *adtA*, whereas, primers CtpA-1 and CtpA-4 (see Section 5.4) were used for amplification of *ctpA*. The thermal cycling parameters were as follows: 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 3 min, and final annealing step of 72 °C for 10 min. Nearly 200 ng of genomic DNA treated or untreated with RNase-free DNase were used in PCR amplifications as additional controls. The RT-PCR and subsequent PCR assays were repeated once to confirm the results. PCR products were size fractionated through a 0.8% agarose gel.

#### 5.6. Growth rates of *B. mallei* strains

Single colonies of the wild type strain ATCC 23344, the *sacB* mutant 23344Δ*sacB*, and the *ctpA* mutant 23344Δ*sacB*Δ*ctpA* were grown at 37 °C for 72 h to stationary phase in 10 ml of TSB-G. These cultures were used to inoculate 25 ml of TSB-G or salt-free LB-G. Cultures were grown at 37 °C and shaken at 200 rpm; Klett readings were recorded every three h in a Klett-Summerson colorimeter (New York, NY), and the cfu/ml were determined by viable plate count.

#### 5.7. Electron microscopy

Fifty microliters of the stationary phase cultures of the wild type, the *sacB* mutant, and the *ctpA* mutant strains were used to inoculate 10 ml of fresh TSB-G. The cultures were grown for 18 h at 37 °C while shaking at 200 rpm. The cells were harvested by centrifugation at 2000 × g for 20 min, washed with PBS, and fixed overnight at 4 °C in formaldehyde–paraformaldehyde in cacodylate buffer [32]. The samples were then processed for thin-section electron microscopy as described by Banai et al. [32]. The sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a JOEL 100 CX-II transmission electron microscope (Zeiss 10C; Carl Zeiss Inc., New York, NY) at ×6300 magnification.

#### 5.8. Preparation of *B. mallei* inoculum stocks

Five microliters of the stationary phase cultures of the wild type, the *sacB* mutant, and the *ctpA* mutant strains were used to inoculate 10 ml of fresh TSB-G. The cultures were grown for 72 h at 37 °C with shaking (200 rpm). The cells were harvested by centrifugation at 2000 × g for 20 min, washed with PBS, and resuspended in 10 ml of PBS. The dilutions of cultures were plated on TSA-G plates to determine the cfu/ml.

#### 5.9. Persistence of recombinant *B. mallei* strains in macrophages

The mouse macrophage-like cell line J774.2 was obtained from the American Type Culture Collection (Manassas, VA). The J774.2 cells were seeded at a density of 5 × 10<sup>5</sup>/ml in Dulbecco's modified essential medium (DMEM) (Sigma-Aldrich, St. Louis, MO) into 24-well tissue culture dishes (2 ml macrophage suspension/well)

and cultured at 37 °C with 5% CO<sub>2</sub> until confluent. The tissue culture medium was removed, 200 µl (10<sup>8</sup> cells) of the *B. mallei* suspension in PBS was added (at a multiplicity of infection of 100 bacteria per macrophage), and the cells were incubated at 37 °C for 4 h. The suspension above the cell monolayer was removed, and the cells were washed three times with PBS. One milliliter of DMEM containing 25 µg of gentamicin was added, and the cells were incubated for 48 h at 37 °C. At various time points (4, 12, and 24 h of incubation), the growth medium was removed, the cells were washed with PBS, and 500 µl of 0.25% sodium deoxycholate was added to lyse the infected macrophages. After 5 min the lysate was diluted in PBS, and the number of viable cells was determined after growth at 37 °C for 72 h on TSA-G plates. Triplicate samples were taken at each time point, and the assay was repeated two times.

#### 5.10. Pathogenicity of *B. mallei* strains in mice

Seven-week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were allowed 1 week of acclimatization. Groups of five mice each were intraperitoneally injected with three different doses (4.4 × 10<sup>5</sup>, 6.6 × 10<sup>5</sup>, and 8.8 × 10<sup>5</sup> cfu/mouse) of each strain: wild type, *sacB* mutant, and *ctpA* mutant. Survival of animals for 36 days post-inoculation was monitored, and abnormal animal behaviors of surviving animals (any huddling or fur ruffling) were recorded. The 50% lethal dose (LD<sub>50</sub>) of the treatment groups was calculated using the Probit.exe program of STAT 2050 server of the University of Guelph, Canada ([http://www.uoguelph.ca/~jhubert/stat2050/software/software\\_2050.html](http://www.uoguelph.ca/~jhubert/stat2050/software/software_2050.html)). The number of animals dying up to 6 days post-inoculation was used in LD<sub>50</sub> calculations. The animals that received wild type or *sacB* strains and survived were sacrificed by exposing to CO<sub>2</sub> on day 36 post-inoculation. Their spleens and livers were homogenized and cultured to determine the presence of the inoculated strain [7].

#### 5.11. Enzyme-linked immunosorbent assay

Blood samples were collected by retro-orbital bleeding from mice injected/inoculated with saline or the *ctpA* mutant, on day 30 post-inoculation/injection. Sera were collected by centrifugation at 3000 × g for 5 min. The heat-killed wild type strain ATCC 23344 suspended in 0.06 M sodium carbonate buffer (pH 9.6) was used to coat polystyrene plates (Nunc 2-69620, Denmark) at approximately 10<sup>7</sup> cells/well. After overnight incubation at 4 °C, the plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T), and the diluted serum samples (1:100 dilution in PBS) from mice inoculated with *B. mallei* strains were added to the wells (100 µl/well). Each serum sample was tested in triplicate. The plates were incubated for 2 h at room temperature. The plates were washed three times with PBS-T, and horseradish peroxidase labeled rabbit anti-mouse IgG (Sigma) diluted at 1:5000 in PBS-T (100 µl/well) was added. After 15 min of incubation at room temperature, the plates were washed three times with PBS-T, and 100 µl of substrate solution (TMB Microwell peroxidase substrate; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. After 10 min of incubation at room temperature, the enzyme reaction was stopped by adding 100 µl of stop solution (0.185 M sulfuric acid), and the A<sub>450</sub> was recorded with a microplate reader (Molecular Devices, Sunnyvale, CA).

#### 5.12. Protection of CD1 mice against lethal challenge with wild type *B. mallei*

At day 36 post-inoculation, those mice that were injected with saline or the *ctpA* mutant and survived were challenged intraperitoneally with 6.6 × 10<sup>5</sup> cfu/mouse (1.1 times the LD<sub>50</sub>) of wild type strain ATCC 23344. Survival of the mice for 15 days

post-challenge was monitored, and abnormal animal behaviors were recorded. On day 15 post-challenge, the surviving animals were killed by CO<sub>2</sub> asphyxiation. Their spleens and livers were homogenized and cultured to determine the presence of *B. mallei*.

#### 5.13. Data analyses

The mean and standard deviation values from ELISA and macrophage assays were calculated using the Microsoft Excel 2001 program (Microsoft Corporation). The data were further analyzed by performing analysis of variance, and the mean cfu counts among treatments were compared using the least-significance pair-wise comparison procedure [40].

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